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ORIGINAL ARTICLE

WILEY Helicobacter

Natural fruits, flowers, honey, and honeybees harbor *Helicobacter pylori*-positive yeasts

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Abstract

Background: For controlling *Helicobacter pylori* infection in humans, its environmental reservoir should be determined. In this study, yeast isolates from an isolated village in Iran were studied for the intracellular occurrence of *H. pylori*.

Materials and Methods: In this study, yeasts were isolated from 29 samples, including oral swabs from villagers (n = 7), flowers and fruits (n = 6), honey and honeybees (n = 12) and miscellaneous samples (4). Yeasts were classified into 12 RFLP groups and identified by amplification of *26S rDNA* and sequencing. DNA extracted from the yeast cells was examined for the presence of *H. pylori* using PCR.

Results: Of the 29 yeasts, 27 were members of different genera of *Ascomycete*. *H. pylori* was detected in 5 of 9 *Candida* (55.5%), 4 of 5 *Komagataella* (80%), 3 of 4 *Pichia* (100%), 2 of 2 *Cytobasidia* (100%), 2 of 2 *Hansenia* (100%), 1 of 1 *Meyerozyma* (100%) and 2 of 3 not sequenced (66.6%) yeasts. Distribution of 19 of 29 (65.5%) *H. pylori*-positive yeasts within 4 groups was as follows: 1 of 7(14.3%) in oral swabs, 5 of 6 (83.3%) in flowers and fruits, 10 of 12 (83.3%) in honey and the bee group and 3 of 4 (75%) in miscellaneous.

Conclusions: Different genera of osmotolerant yeasts from flowers, fruits, honey, and honeybees contained *H. pylori* in their vacuole. High frequency of *H. pylori*-positive yeasts in these samples might be related to their high sugar content. Insects such as honeybees that facilitate transfer and easy access of these yeasts to nectars serve as the main reservoirs of these yeasts, playing an important role in their protection and dispersal. Accordingly, *H. pylori* inside these yeasts can be carried by honeybees to different sugar- and nutrient-rich environments. Sugar-rich environments and honeybees play an important role in distribution of *H. pylori*-positive yeasts in nature.

KEYWORDS

distribute, honeybees, intracellular *Helicobacter pylori*, osmotolerant yeasts, sugar-rich environments

1 | INTRODUCTION

It is well accepted that *Helicobacter pylori* plays a significant role in gastric diseases including gastritis, peptic ulcers, gastric cancer, and MALT lymphoma.[1] Person-to-person contact has been regarded as the most common route of *H. pylori* infection.[2] Moreover, as *H. pylori* is a gastric colonizer, ingestion of contaminated food and water[3] has also been suggested as an important route of *H. pylori* entry into the human stomach. However, there is no evidence to show survival of *H. pylori* in the environment outside human stomach such as oral

cavity,[4] water,[5] or food.[6] Accordingly, an important prophylactic measurement to control *H. pylori* entry into the human stomach could be the determination of its environmental source(s). In our previous studies, it was demonstrated that vacuole of *Candida* species which is the storage site for plenty of nutrients[7] could serve as a niche for *H. pylori*, outside the human stomach. The free-living and ubiquitous *Candida* yeast which occur in nature, foods, as well as on human body surfaces could accommodate *H. pylori* in its vacuole, protect it against external stresses, and act as a safe vehicle for spreading the bacterium in the environment and within human hosts.[8-11]

Yeasts are unicellular fungi which prefer nutrient-rich environments, exhibit rapid growth and multiplication in high concentrations of simple sugars, and exhibit remarkable metabolic diversity.[12] Yeasts have been exploited by humans since ancient times when fermentation was the primary method to prepare and preserve foods.[13] The origin of fermentative yeasts is not well known. According to reports, development of a fermentative lifestyle in yeasts was concomitant with the evolution and abundance of a variety of flowering plants that possess high sugar.[14,15] As fermentative yeasts have been isolated from insects, it is believed that these symbiotic yeasts have been transported to flowers and fruits by insects.[16,17] Association of yeasts with insects could indicate their evolutionary relationship in which yeasts provide services to insects and vice versa.[18,19] Insects protect their symbiotic yeasts, disperse their spores, and facilitate their outbreeding.[20] Furthermore, insects maintain their fungal symbionts by ingestion and reingestion of feces, ingestion of contaminated food and inoculation of their eggs.[21,22] Yeasts, in turn, can serve insects as nutrients, detoxify toxic compounds, protect them against stresses, and facilitate their chemical communication by producing volatile compounds.[23] Yeasts can also be used by insects as a source of intermediate precursor for ergosterol biosynthesis.[24] Among insects, honeybees as the major pollinators of a variety of plant species that play a crucial role in maintenance of biodiversity of different ecosystems[25] ingest yeasts while foraging on flowers. Worker bees and the queen maintain their ingested yeasts during cold seasons by flying to warm regions and inoculate them into new flowers during pollination at the beginning of spring.[26]

Honey, a natural product of bees, is exclusively made for feeding their larvae to support their growth, development, and health. Accordingly, honey should have remarkable nutritional and antimicrobial properties. Indeed, honey has been appreciated by humans for thousands of years as a sweet and flavorful food material that aids the growth and development of humans, reinforces their health,[27] and serves as a traditional remedy for treatment of a wide range of diseases.[28,29] All these benefits of honey are due to properties such as antioxidant,[30] anti-inflammatory,[31] tissue repair,[32] and energy provision.[33] The main components of honey are sugars (75%) and water (18%-21%). Other components are proteins (enzymes), organic acids, vitamins (vitamin B6, thiamin, niacin, riboflavin, and pantothenic acid), minerals (calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc), pigments, phenolic compounds, and volatile compounds.[34-36] It appears that 200 components act in synergy to confer the unique and sophisticated properties of honey.[37] One important reason for the efficacy of honey in the traditional treatment of a number of diseases relates to its antimicrobial activity against a wide range of microorganisms: bacteria,[38] fungi,[39] viruses,[40] and parasites.[41] It has been reported that hydrogen peroxide,[42] low moisture and high concentration of sugar,[43] and an acidic pH (3.2-4.5)[44] all contribute to antimicrobial properties of honey. However, a considerable number of reports indicate that honey has its own microbial community, mainly composed of yeast and bacteria.[45] Nectars, fruits, and honeydews (sugar-rich and sticky secretions of plant-feeding insects or secretions from the living parts of plants)[44] on which bees forage are the main sources of honey microbiome.[46] These substrates with high contents of sugar and nutrients accommodate a diverse range of microorganisms,[47] mainly yeasts (>10^6/mL)[48] and bacteria (>10^7/mL).[49] Evidence would suggest that bacteria and yeasts in nectar establish nutritional interactions that help them to tolerate the high concentrations of sugar.[50,51]

The aim of this study was to determine whether yeasts that colonize natural and intact environments, far from modern human activities, could harbor *H. pylori* inside their vacuole. Furthermore, we wished to determine to which genera the yeasts belonged and whether genera other than *Candida* also contain *H. pylori*. A natural and isolated village, Sorkhabad, in Alborz Mountains of north Iran, was selected for sample collection from the oral cavity of villagers, natural flowers and fruits, local honey, beehives, and captured bees. Isolated yeasts were identified by PCR-restriction fragment length polymorphism (RFLP), followed by sequencing of *26S rDNA*. Yeast isolates were examined by light and fluorescent microscopes to observe the bacterial bodies (BBs) inside the yeasts vacuole. PCR was used for detection of *H. pylori*-specific *16S rDNA*, by designed primers.

2 | MATERIALS AND METHODS

2.1 | Collection and culture of samples

An isolated village, Sorkhabad, located in an intact region of Alborz Mountains, north of Iran, was selected as a natural place far from human activities, for isolation of yeasts from different sites. The 43 collected samples were classified into 4 groups: oral swabs (12 oral swabs from villagers), flowers and fruits (10 samples from natural flowers and fruits), honey and the bee group (17 samples from bees' body and honey), and miscellaneous (4 samples from different urban sources).

2.1.1 | Oral swabs

Sterile cotton swabs were used for collection of oral samples from 12 of 30 villagers who were available at the time of sampling: 7 male and 5 female, with the age range of 10-90 years. Oral swabs were transported to microbiology laboratory in semisolid physiological saline (0.85% NaCl and 0.1% agar in distilled water) and cultured on yeast extract-glucose chloramphenicol (YGC) agar (Merck, Germany) and examined for the yeast growth after 24-48 hours incubation at 30°C.

2.1.2 | Wild flowers and fruits

One gram of whole flowers or fruits: 2 *Cirsium arvense*, 1 plum (*Prunus* subgroup *Prunus*) flower, 2 *Menthe pulegium*,[1,2] 1 *Vicia villosaroth*, 1 *Erysimum repandum*, and 1 *Trifolium pratense*, 1 plum (*Prunus* subgroup *Prunus*) fruit, and 1 quince (*Cydonia oblonga*) fruit was inoculated into 3 mL of brain heart infusion (BHI) broth (Merck, Germany) and incubated at 30°C for 7-10 days. A 50-µL volume of each BHI broth was surface inoculated on YGC agar and observed for the yeast growth after 24-48 hours incubation at 30°C.

2.1.3 | Honey and honeybees

Sterile forceps, glass Petri dishes, and stereomicroscope were used for dissecting the captured bees (*Apis mellifera*), isolation of the intestine, and detaching the stingers. A sterile mortar was used for pulverizing the bees. To slow down the movement of the bees prior to dissection and pulverizing, they were incubated at 4°C for 3 minutes. Tubes of 3-ml BHI broth were inoculated with up to 1 gram of different parts of bees' body: head, mouth, stinger, intestine, anus, as well as a small natural beehive, and a pure local honey. Furthermore, intact body of one bee and pulverized body of another bee were inoculated into tubes with 3-ml BHI broth. After 7-10 days incubation at 30°C, a 50-µL volume of each BHI broth was surface inoculated on YGC agar. Plates were observed for yeast growth after 24-48 hours incubation at 30°C. Two actively moving bees (a, and b) were also left on the surface of 2 YGC agar plates at room temperature, in order to allow sufficient time for the bees' body to have contact with the medium and allow transfer of the yeasts onto the medium. After 24 hours, bees were removed, and plates were examined for yeast growth up to 2 weeks incubation at 30°C.

2.1.4 | Miscellaneous samples

These samples were collected from the urban area of Tehran, Iran, for comparing their yeast isolates with those of the natural village for the occurrence of intracellular *H. pylori*. A social wasp (*Vespula germanica*) gut, a pulverized whole *Drosophila melanogaster*, a pulverized whole ant (*Formica rufa*), and one gram of Gazangabin (honeydew collected from a plant, *Astragalus adscendens* which grows in central Iran) were inoculated into 3-ml BHI broth and treated as mentioned above.

2.2 | Isolation of yeasts

All the YGC agar plates were examined after 24-48 hours for yeast growth. Colonies from the 29 yeast-positive culture plates were Gram-stained and examined by the light microscope for observing typical morphology of yeasts. A single colony was selected from each yeast-positive culture and subcultured on YGC agar for more than 10 times, to ensure the elimination of extracellular bacteria. Pure cultures of yeasts were used for their molecular identification and detection of *H. pylori*-specific *16S rDNA*.

2.3 | Molecular identification of yeasts

2.3.1 | PCR-restriction fragment length polymorphism (RFLP) of internal transcribed spacer (ITS) region in 5.8S rDNA

Fresh cultures of 29 yeasts were used for extraction of DNA according to Sambrook.[52] Primers used to amplify the ITS region were ITS1(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5-TCCTCCGCTTATTGATATGC-3′).[53] Amplification was performed with 500-1000 ng yeast DNA as a template and initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. PCR products were electrophoresed, using 1% agarose gel in Tris/Borate/EDTA (TBE, 0.5 X), and digested without further purification, using restriction endonucleases Hha I, Hae III, and Hinf I (Promega, USA and Bioron, Germany). Restriction fragments were electrophoresed using 2% agarose gel, and the size of fragments was determined according to 50-1500 bp molecular ladder (CinnaGen, Iran). Yeasts were classified into 12 groups according to their RFLP pattern (Table 1).

2.3.2 | Amplification and sequencing of 26S rDNA

Amplification of D1/D2 region of *26S rDNA* from 29 yeasts was carried out, using primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGC-3′).[53] PCR was performed with 500-1000 ng of yeast DNA and initial denaturation at 94°C for 1min, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 50°C for 1 minutes, and extension at 72°C for 2 minutes with final extension of 72°C for 5 minutes. The PCR products were electrophoresed on a 1% agarose gel, and their size was determined as 600 bp, based on comparison with molecular ladder (50-1500 bp) (CinnaGen, Iran). PCR products of representative yeasts from 9 RFLP groups (groups: 2-7, 9, 10, and 12) were purified and sequenced. The BLAST-querying program was used to match the sequences with published sequences of yeasts in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

2.4 | Observation of bacterial bodies (BBs) inside the vacuole of yeasts by light microscopy and fluorescent microscopy

Wet mounts were prepared from fresh cultures of yeasts on YGC agar and examined by light microscopy to observe the fast-moving BBs inside the vacuole of yeasts. Furthermore, to determine whether BBs were alive, a fresh culture of the yeast isolate from a beehive (*Hanseniaspora uvarum*) was used for staining with LIVE/DEAD BacLight Bacterial Viability Kit L-7012 (Invitrogen, Eugene, Oregon, USA) and fluorescent microscopy. A suspension of yeast cells was stained with equal volumes of SYTO 9 and propidium iodide. After incubation in the dark, a wet mount was examined by the fluorescent microscope (Olympus, Tokyo, Japan). Images were taken from moving BBs inside the yeasts' vacuole at 4-second time intervals.

TABLE 1 Classification of 29 yeast isolates from natural sources in Sorkhabad village according to their PCR-RLFP pattern

RFLP Group	No. of yeast isolates	Sources	PCR Product	Restriction fragment (bp)		
				HhaI	HaeIII	HinfI
1	1	*M. pulegium2*	400	220, 180	400	200
2	1	*P. subg. Prunus* (fruit)	750, 500, 400	400, 350, 220, 180	750, 400	350, 200, 100, 90
3	5	bee stinger 1, bee stinger 2, bee stinger 3, bee intestine 3, *C. arvense*	450	450	450	250, 200
4	1	*P. subg. Prunus* (flower)	500	200, 170, 70	400, 100	200, 150, 140
5	3	oral swab 5, oral swab 6, oral swab7	550	290, 260	460, 90	280, 270
6	2	moving bee b, *M. pulegium*1	600	600	600	350, 250
7	2	pulverized *F. rufa*, moving bee a	600	350, 250	400, 120, 80	320, 280
8	1	oral swab 8	600	300, 250, 60	400, 130, 70	300, 180, 120
9	2	*C. oblonga* (fruit), beehive	700, 350	400, 350, 100	700, 360	350, 200, 150, 100, 70, 50
10	9	*V. germanica* gut, pulverized bee, honeybee, local honey, bee intestine 1, bee intestine 2, oral swab 4, oral swab 11, oral swab12	800-900	400, 160, 140	680, 220	360, 250
11	1	*A. adscendens* (honeydew, gazangabin)	400	220, 80	400	200, 120, 80
12	1	pulverized *D. melanogaster*	600	300, 250, 200, 100, 50	400, 120, 80	320, 280, 200

2.5 | Detection of *Helicobacter pylori* -specific 16S rDNA in yeasts

DNA extracted from 29 yeasts was examined for the presence of *Helicobacter pylori*-specific *16SrDNA* using the HP1 and HP2 primers.[54] Recruited primers were HP1 (5′-GCAATCAGCGTCAGTAATGTTC-3′) and HP2 (5′-GCTAAGAGATCAGCCTATGTCC-3′). A clinical isolate of *H. pylori* which was previously identified by amplification and sequencing of *H. pylori*-specific *16S rDNA* was used as a control. Amplification started with an initial denaturation at 94°C for 3 minutes, followed by 33 cycles of 94°C for 45 seconds, 57°C for 1 minute and 72°C for 1 minute, and the final extension of 72°C for 5 minutes. PCR products were electrophoresed and their size determined, using a 50-1500 bp DNA ladder (CinnaGen, Iran).

3 | RESULTS

3.1 | Isolation of yeasts

Microscopic examination of Gram-stained smears of yeasts colonies on YGC agar showed typical yeast morphology. Of 43 samples, 29 were positive for yeast. Yeast-positive samples included 7 of 12 (58.3%) oral swabs, 6 of 10 (60%) flowers and fruits, 12 of 17 (70.6%) honey and the bee group, and 4 of 4 (100%) miscellaneous samples (Table 1).

3.2 | Molecular identification of yeasts

Amplification of the ITS region in the *5.8S rDNA* of 29 yeasts revealed bands with the size between 400 and 900 bp which were digested with restriction endonucleases. According to their PCR-RFLP patterns, the yeasts were then classified into 12 groups. The RFLP groups 10 and 3 had the highest number of yeasts, with 9 *Candida glabrata* and 5 *Komagatella pastoris* being identified in each group, respectively (Table 1). Electrophoresis of the amplified products of the D1/D2 region of the *26S rDNA* from 29 yeasts showed bands with the size of 600 bp (Figure 1). Sequence analysis of 9 of the 12 RFLP groups showed 99% similarity to 9 different yeasts species in the GenBank database. In the oral swabs group, three yeast isolates were identified as *Candida albicans* (5, 6, and 7), and 3 isolates were identified as *Candida glabrata* (Table 2). In the flower and fruit group, except for the yeast isolate from the flower *M. pulegium* 2 which was not sequenced, other yeast isolates were identified as *K. pastoris*, *Cystobasidium sloofiae*, *Hanseniaspora uvarum*, *Pichia kudriavzevii*, *and Galactomyces candidum* (Table 3). In the honey and the bee group, yeast isolates from a local honey sample, a pulverized bee, a whole honeybee, and two bee intestines[1,2] were identified as *C. glabrata*. Yeast isolates from 3 different bee stingers (1, 2, and 3) and bee intestine 3 were identified as *K. pastoris*. Yeast isolates from 2 different moving bees (a, b) were identified as *P. guilliermondii* and *C. slooffiae*. The one yeast isolate from a beehive was identified as *H. uvarum*

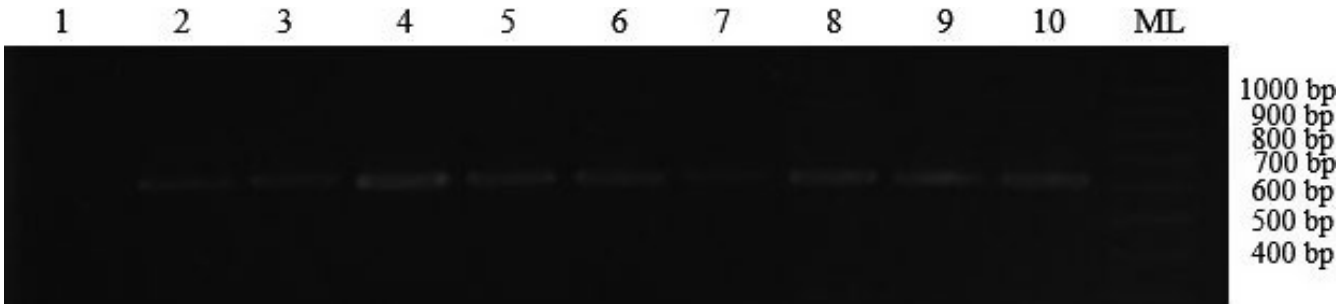

FIGURE 1 Detection of *26S rDNA* in yeast isolates from the honey and bee group. The PCR products of 600 bp amplified from 9 yeasts (lanes 2-10) are shown. Negative control (lane1), molecular ladder, ML (Lane 11)

TABLE 2 Frequency of *Helicobacter pylori -16S rDNA* in 7 yeast isolates from oral swabs group

Samples (n = 7)	RFLP group	*26S rDNA* D1/D2 sequencing result	*H. pylori-16S rDNA*
Oral swab 4	10	*Candida glabrata*	+
Oral swab 5	5	*Candida albicans*	–
Oral swab 6	5	*Candida albicans*	–
Oral swab 7	5	*Candida albicans*	–
Oral swab 8	8	NS	–
Oral swab 11	10	*Candida glabrata*	–
Oral swab 12	10	*Candida glabrata*	–

NS, Not sequenced.

(Table 4). In the miscellaneous group, the yeast isolate from the wasp gut was identified as *C. glabrata*, the yeast isolate from the pulverized *D. melanogaster Meyerozyma guilliermondii* as *P. guilliermondii*, and the isolate from a pulverized ant *P. guilliermondii* (Table 5). (The yeast isolate cultured from *Astragalus ascendens* was not sequenced.) Most of the yeasts (27/29) were members of *Ascomycetes*, and the remaining 2 *Basidiomycetes*; *C. slooffiae*.

3.3 | Light and fluorescent microscopy for observation of BBs inside the yeasts

Microscopic examination of wet mounts prepared from fresh cultures of 29 yeasts showed the occurrence of BBs inside the vacuole of all yeasts cells (data not shown). Fluorescent microscopy of the yeast isolate from a beehive (*Hanseniaspora uvarum*) showed that BBs inside the vacuole of yeasts cells were green and thus alive (Figure 2A-C). Images taken at 4-second time intervals displayed rapid movement of BBs inside the vacuole of yeast's cells (Figure 3).

3.4 | Detection of *Helicobacter pylori -16S rDNA* in yeasts

Amplification products of *Helicobacter pylori* -specific *16S rDNA* (521 bp) were detected in 19/29 (65%) of yeasts (Figure 4). In the oral swabs group, only one *C.glabrata* (14.3%) was *H. pylori*-positive (Table 2). In the flower and fruit group, 5 of 6 yeasts (83.3%): *K. pastoris*, *C. slooffiae*, *H. uvarum*, *P. kudriavzevii*, and one isolate which was not sequenced (NS), were *H. pylori*-positive (Table 3). In the honey and the bee group, 10 of 12 yeasts (83.3%) were positive for the occurrence of *H. pylori*: *C. glabrata* (x4), *K. pastoris* (x3), *P. guilliermondii*, *C. slooffiae*, and *H. uvarum* (Table 4). In the miscellaneous group, 3 of 4 yeasts (75%) were positive for *H. pylori*: *P. guilliermondii*, *M. guilliermondii*, and one which was not sequenced (Table 5). Comparison of the 4 yeast-positive groups showed the frequency of *H. pylori* to be the highest in the honey and bee group (83.3%), flower and fruit group (83.3%) followed by the miscellaneous group (75%) and the oral swab group (14.3%) (Figure 5).

4 | DISCUSSION

Yeasts that thrive on sugar-rich environments have been exploited by humans for production of fermented foods, since antiquity.[55]

TABLE 3 Frequency of *Helicobacter pylori -16S rDNA* in 6 yeast isolates from flower and fruit group

Samples (n = 6)	RFLP group	*26S rDNA* D1/D2 sequencing result	*H. pylori-16S rDNA*
C. arvense	3	*Komagataella pastoris*	+
M. pulegium1	6	*Cystobasidium slooffiae*	+
M. pulegium2	1	NS	+
C. oblonga (quince fruit)	9	*Hanseniaspora uvarum*	+
P. subg. Prunus (plum flower)	4	*Pichia kudrizavii*	+
P. subg. Prunus (plum fruit)	2	*Galactomyces candidum*	-

NS, Not sequenced.

TABLE 4 Frequency of *Helicobacter pylori -16S rDNA* in 12 yeast isolates from the honey and bee group

Samples (n = 12)	RFLP group	*26S rDNA* D1/D2 sequencing result	*H. pylori-16S rDNA*
Pulverized bee	10	*Candida glabrata*	+
Honeybee	10	*Candida glabrata*	+
Local honey	10	*Candida glabrata*	+
Moving bee (a)	7	*Pichia guiliermondii*	+
Moving bee (b)	6	*Cystobasidium slooffiae*	+
Bee stinger 1	3	*Komagataella pastoris*	+
Bee stinger 2	3	*Komagataella pastoris*	+
Bee stinger 3	3	*Komagataella pastoris*	+
Bee intestine 1	10	*Candida glabrata*	+
Bee intestine 2	10	*Candida glabrata*	–
Bee intestine 3	3	*Komagataella pastoris*	–
Beehive	9	*Hanseniaspora uvarum*	+

TABLE 5 Frequency of *Helicobacter pylori -16S rDNA* in 4 yeast isolates from miscellaneous group

Samples (n = 4)	RFLP group	*26S rDNA* D1/D2 sequencing result	*H. pylori-16S rDNA*
V. germanica gut	10	*Candida glabrata*	–
pulverized *D. melanogaster*	12	*Meyerozyma quilliermondii*	+
pulverized *F. rufa*	7	*Pichia guiliermondii*	+
A. adscendens	11	NS	+

NS, not sequenced.

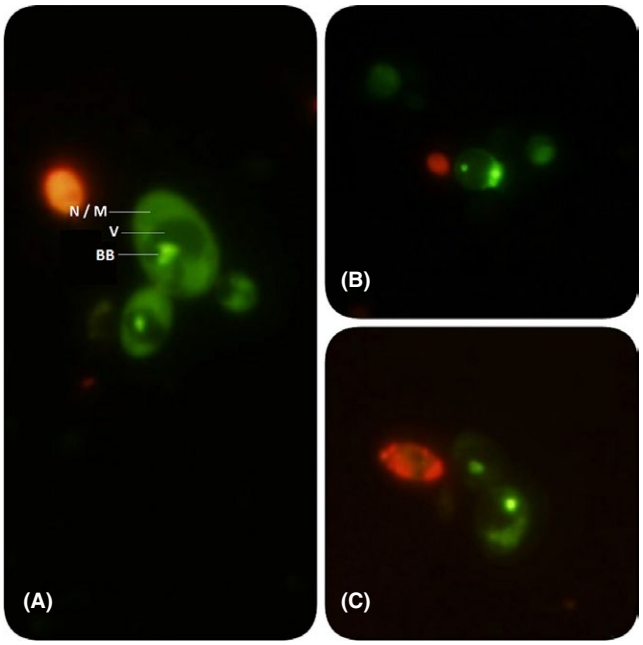

FIGURE 2 Fluorescent microscopy of bacterial bodies (BB) inside the vacuole of a yeast isolate from a beehive (*Hanseniaspora uvarum*). BB inside the dark vacuoles (V) appear green indicating that they are alive (A, B, and C). The green areas outside the vacuoles are related to nucleus (N) or mitochondria (M). (Original magnification ×1000)

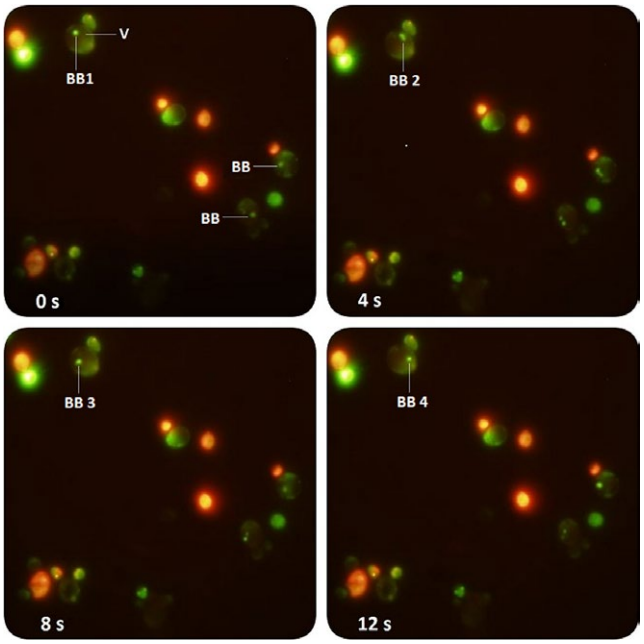

FIGURE 3 Fluorescent microscopy of fast-moving bacterial bodies (BB) inside the vacuole (V) of a yeast isolate from a beehive (*Hanseniaspora uvarum*). Images taken at approximately 4-second time intervals (0, 4, 8, and 12 s) show different locations of moving BB (BB 1, 2, 3, and 4) inside the yeast's vacuole. (Original magnification ×1000)

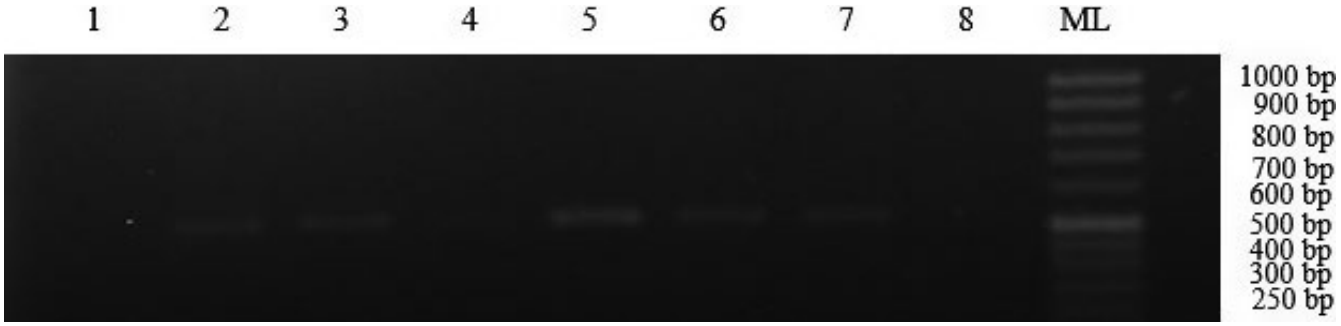

FIGURE 4 Amplification of *Helicobacter pylori -16S rDNA* from 6 yeast isolates selected from the honey and bee group. A PCR product of 521 bp was amplified from 4 yeasts: *Hanseniaspora uvarum*, *Candida glabrata*, *Komagetella pastoris*, and *Pichia guiliermondii* (lanes 2, 3, 5, and 6). Negative amplification results: *Candida glabrata* and *Komagetella pastoris* (lanes 1 and 4), positive control (lane 7), negative control (lane 8), and molecular ladder, ML (lane 9)

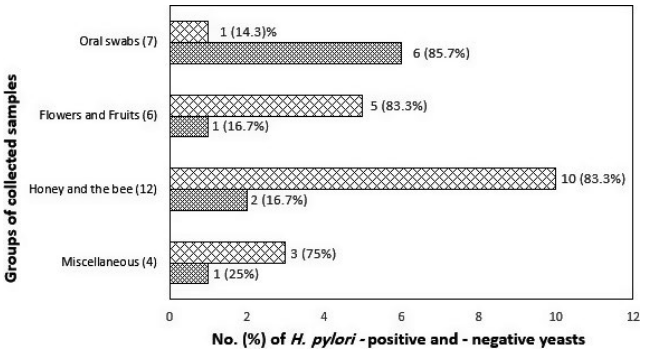

FIGURE 5 Frequency of *Helicobacter pylori*-positive and -negative yeasts in 4 groups of collected samples; oral swabs, flowers and fruits, honey and the bee, and miscellaneous. ▨ *H. pylori*-positive, ▤ *H. pylori*-negative

Reports show that yeasts first entered human life in association with plants.[17] Insects attracted to sweet floral nectars and fruits have been shown to carry yeasts from soil to plants.[16,17] Given this, insect-associated yeasts have easy access to sugar- and nutrient-rich nectars, thus establishing the dominant microflora of flowers and fruits.[18] A large body of evidence describes the mutual interactions between insects and yeasts with a profound impact on the evolution of both partners.[18,19] Yeasts are ubiquitous and free-living microorganisms that occur in soil, water, and food[56] as well as on human body surfaces.[57] This wide spectrum of habitats indicates that yeasts have evolved to adapt to a wide range of environmental conditions,[58-60] including low water activity and high concentrations of sugars.[59] Nectar and honey with low water and high sugar contents while inhibiting microbial growth and multiplication,[45,61,62] accommodate different osmotolerant microorganisms.[45,47] Yeasts that display rapid growth and multiplication in sugar-rich nectars serve as rich sources of proteins and vitamins for nectar-feeding insects, especially honeybees.[63] Studies would suggest that honeybees as the major pollinators transfer yeasts from their body to a variety of flowers while foraging on the pollen and nectar.[64] During foraging in warm seasons, yeasts are transferred to the bees' digestive system and then to the beehive where they are protected in cold seasons and transferred to new larvae, in the spring.[65] Accordingly, honeybees have been recognized as the natural sources of yeasts that play an important role in their distribution in the environment and outbreeding.[20,66]

In this study, samples were collected from Sorkhabad, a natural and intact village in the Alborz Mountains in northern Iran. The 43 samples collected in this study were classified into four groups: oral swabs, fruits and flowers, honey and the bee, and miscellaneous. A single colony was selected from each of the 29 yeast-positive cultures and subcultured on the YGC to eliminate any possible contamination with extracellular bacteria. Yeast isolates were classified into 12 groups according to their RFLP profiles. Sequencing of the amplified products obtained from the yeast *26S rDNA* revealed that the RFLP groups 10 and 3 contained the highest number of yeasts these being 9 *C. glabrata* and 5 *K. pastoris*, respectively. Of the 29 yeast isolates, 27 (93%) were members of the *Ascomycete* and 2 (7%) belonged to the *Basidiomycete* group. Sugar tolerant yeasts are common inhabitants of floral nectar[67] and raw honey[68] and belong to the osmotolerant and fermentative *Ascomycete* yeasts. This group of yeasts being more adapted to the conditions in nectar than the *Basidiomycete* yeasts[69] and can reach high densities in nectar.[26] Previous studies have reported the frequent isolation of *Candida* species from sugar-rich nectars, honey and beehives,[68] for example, *C. magnoliae* has been reported to be transported in pollen to the comb cells of the hive,[70] *C. bombicola* from the nectar and pollen,[71] and *C. etchellsii* from bees.[72] Other yeasts such as *Saccharomyces cerevisiae* have been isolated from *D. melanogaster*,[73] hibernating wasps[65] and beehives[74] and *P. guilliermondii* from raw honey.[68] Members of the *Ascomycetes* have also been reported as the main yeast isolates from fruits and blossoms of plum, apple, and pear trees.[75]

Fluorescent microscopic observation showed that BBs inside the yeast vacuole were alive. Out of 29 yeasts, 19 were positive for *H. pylori 16s rDNA*. The identity of BBs inside the remaining 10 yeasts needs to be elucidated. Five of 9 *C. glabrata* yeasts (55.5%) and 4 of 5 *K. pastoris* (80%) were positive for *H. pylori*. The remaining yeast isolates that contained *H. pylori* were as follows: 4 of 4 *Pichia* (100%), 2 of 2 *Cytobasidia* (100%), 2 of 2 *Hansenia* (100%), 1 of 1 *Meyerozyma*

(100%), and 2 of 3 yeasts that were not sequenced (66.6%). These results indicate that the intracellular occurrence of *H. pylori* inside yeast cells is not related to a particular genus and may occur in different genera of yeasts. The frequency of *H. pylori*-positive yeasts was considerably higher in flowers and fruits (83.3%), honey and honeybees (83.3%), and in the miscellaneous (75%) groups as compared with those in the oral swabs group (14.3%). This finding might be related to the high sugar content of these three habitats.

Sugar-rich habitats such as nectar contain >90% sugar, amino acids, organic acids, lipids, essential oils, polysaccharides, vitamins, antioxidants, and secondary metabolites[76-78] which potentially could support growth and multiplication of yeasts. However, these habitats exert antibacterial effects due to osmotic pressure,[26,79] reactive oxygen derivatives,[80] and phenolic compounds.[81] The isolation of yeasts from floral nectar has been reported by a large number of studies[26,63,69,82-85] compared with those on isolation of bacteria,[49,86] indicating that yeasts are more abundant in sugar-rich habitats than bacteria and exhibit fast growth and multiplication in such environments.[63] Furthermore, investigators have reported the inhibitory effect of different brands of honey on *H. pylori* due to osmotic effects,[87] hydrogen peroxide,[88] or other inhibitory compounds.[89,90] The osmotic effect of honey appears to be the most important parameter for the killing *H. pylori*.[87] In this regard, carbohydrate solutions of ≥15% (v/v) inhibited 100% of *H. pylori* cells.[87] Accordingly, bacteria[26,79-81] including *H. pylori*[87] do not survive in sugar-rich environments. However, the intracellular establishment of *H. pylori* or probably other bacteria inside the vacuole of osmotolerant yeast could serve as a sophisticated strategy for bacterial adaption to these stressful conditions. The results of this study showed that 19 of 29 (65.5%) osmotolerant yeasts carried *H. pylori* in their vacuole. These yeasts were members of 6 genera that inhabit soil, water, and plants as their natural niche. A number of these yeasts, including *Candida*[91] and *Meyerozyma*,[92] have been reported as emerging opportunistic pathogens. Whether these yeasts can release *H. pylori* once ingested remains to be elucidated.[93] Based on our findings, it is plausible to propose that osmotolerant yeasts in nectar and honey which have been transferred from soil to sugar-rich environments by insects could harbor *H. pylori*. These yeasts might serve as the environmental source of *H. pylori* that while protecting the bacterium from being killed by osmotic shock,[87] distribute it within flowers, fruits, honeydews, and honey while carried by insects such as honeybees.

In our previous studies, it was proposed that gastric,[8] oral,[9,10] vaginal,[94] and foodborne [93] yeasts could serve as reservoirs of *H. pylori*. Results of this study propose that *H. pylori*-positive yeasts originate from soil where they are picked by bees and transferred to floral nectars, fruits, honeydews, beehive, and honey. Honeybees appear to play a crucial role in protection, dispersal, and outbreeding of these yeasts. Yeasts can degrade cellulose, the most abundant carbon source on earth, and other complex polysaccharides[95] to easily consumable glucose that serves as a carbon and energy source for microorganisms in soil.[96] Yeasts also provide their insect hosts with essential nutrients: B vitamins, proteins, nitrogen, amino acids, and trace metals.[20,23] Moreover, they are used by insects as an important source of esterols that are required for their cell membranes.[97] In this regard, one important reason for the occurrence of *H. pylori* inside the yeast's vacuole which contains high amount of ergosterol[98] could allow the bacterium easy access to nutrients, in particular esterols which are the precursor of cholesterol, which could be used to construct its cell membrane.[99] In the other words, provision of cholesterol, as the precursor of ergosterol, could be the mutual benefit of *H. pylori* to yeast. Give and take of esterols seems to be that evolutionary thread running within the three partners: insect, yeast, and *H. pylori*, a perfect example of innovation through symbiosis.

DISCLOSURES

The authors have no conflict of interest to declare.

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